Evaluation of an Enzyme-Linked Binding Protein Assay for Hyaluronic Acid and Concentrations in Hepatitis C Infected Patients

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ABSTRACT (Updated)

Serological hyaluronic acid (HA) has been proposed as a noninvasive alternative to liver biopsy for assessing the extent of liver fibrosis. Liver biopsy correctly identifies hepatic disease in about 65 to 75% of cases, being strongly dependent on the length of the biopsy obtained. Furthermore, hepatic fibrosis is often not distributed homogeneously throughout the liver. In contrast, studies suggest HA to have better sensitivity and specificity with areas under receiver operating curves of 0.86 and 0.92, and negative predictive values of 93% and 99% for fibrosis and cirrhosis respectively. Moreover, the non-uniform distribution of hepatic fibrotic tissue does not affect HA results. In this study, we evaluated and validated a commercially available enzyme-linked binding protein assay for measuring HA. We also investigated serum/plasma HA concentrations in subjects having elevated levels of hepatitis C virus (HCV) RNA.

The HA assay (Corgenix Inc., Westminster, CO) is a spectrophotometric sandwich protein binding assay in microplate format. The test utilizes a highly specific HA binding protein (HABP) coated to the microwell surface to capture HA. An enzyme conjugated version of HAPB is subsequently used to detect the HA in the sample. The assay uses six calibrators with final results expressed as ng HA/mL.

Serum and/or plasma samples were stored and assayed according to the kit manufacturer's instructions. The assay's limit of detection was 8 ng/mL resulting in an analytical measurement range of 8 - 800 ng/mL. A linearity study spanning this range generated a slope of 1.008, intercept of 13.64 and R^2 of 0.998 (n = 7). The within-run precision at three levels (n = 8) was determined to be 29 ± 0.8, 138 ± 3.4 and 546 ± 6.7 ng/mL with CVs of 2.7, 2.5 and 1.2% respectively. Between-run precision at three levels (n = 7) resulted in values of 44 ± 3.9, 87 ± 7.6 and 459 ± 29.3 ng/mL generating CVs of 8.8, 8.5 and 6.4% respectively. A correlation study using samples previously assayed at Corgenix produced a slope, intercept and R^2 of 0.957, –0.56 and 0.993 respectively as analyzed by Deming Regression (n = 22, range 29 - 866 ng/mL). Utilizing donations from 122 healthy individuals, an upper 97.5% reference limit of 54 ng/mL was established. HA was found stable for 24 hours at room temperature, and a minimum of two weeks at 4 °C. Deidentified serum or plasma samples from patients with HCV infection were assayed for HA. These patients had HCV RNA levels greater than log 2.3 IU/mL as previously measured by real-time polymerase chain reaction. Of 68 specimens, 53% (36) were found to have elevated HA, with concentrations greater than 37 ng/mL. HA concentration did not correlate with HCV RNA level.

In conclusion, the Corgenix HA test kit has shown acceptable performance characteristics for quantifying HA. Although the stages of liver fibrosis for the HCV infected subjects in this study were unavailable, the large percentage having elevated HA supports previous studies suggesting the possible use of HA in assessing liver fibrosis and/or cirrhosis in lieu of liver biopsy.

INTRODUCTION

Hyaluronic acid (HA), known also as hyaluronan or hyaluronate, is a glycosaminoglycan constructed of repeating sequences of *β*-(1-4)-glucoronic acid and *β*-(1-3)-N-acetylglucosamine moieties. This high molecular weight polysaccharide can vary in length from approximately 10 to more than 1000 of these disaccharide units, with each dimer having a molecular weight of approximately 450 daltons (1).

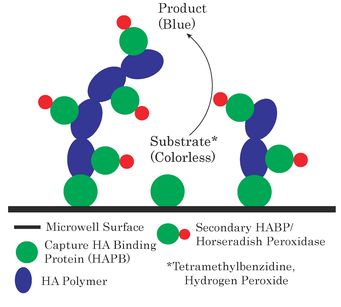
Produced mainly by fibroblasts and other specialized connective tissue cells, HA is widely distributed throughout the body. It has been found to have a structural role in the connective tissue matrix, to participate in cell-to-cell interactions, and exist as a free molecule in synovial fluid and plasma (2-7). The molecular weight also varies depending on the specific location. For example, averages of 220, 2,000 and 7,000 kD are found in lung, cartilage and synovial fluid respectively (7-9). In addition, high HA concentrations in synovial fluid function in water retention and lubrication of the joint (2). HA has also been demonstrated to increase rapidly in response to injuries (10-12).

HA serum levels are maintained by both the liver and kidneys. Removal is dependent upon specific receptors present in sinusoidal endothelial cells of the liver and by the enzymatic action of hyaluronidase (13-16). In liver diseases involving cirrhosis and fibrosis, increased HA levels are caused by decreased hepatic removal possibly due to competition for these receptors, and/or increased HA production during liver inflammation (17-19). Consequently, HA appears to correlate better with the degree of liver damage than conventional tests such as alkaline phosphatase, bilirubin, laminin, tissue growth factor, procollagen III N-peptide and others (13,18,20-26). Therefore, serum HA levels may be useful in distinguishing liver cirrhosis from non-cirrhosis, assessing the extent of liver fibrosis, and for monitoring liver function in subjects suffering hepatitis and/or alcoholic liver diseases (21,24,27-31). In addition, serum HA has been proposed as an early marker of liver damage from toxic agents including acetaminophen, ethanol and bacterial lipopolysaccharide (30,33).

Perhaps the greatest value of HA may be the potential to exclude patients with extensive fibrosis and/or cirrhosis (13). For example, a low HA level has been demonstrated to rule out patients with significant cirrhosis and fibrosis with predictive values of 99 and 93% respectively, thus reducing the need for liver biopsy (28). Moreover, the decision to perform a liver biopsy is not trivial and may lead to significant complications. Furthermore, a needle biopsy only removes approximately 1/50,000th of the liver, which can result in a significant sampling error (13). Therefore, the advantages of a noninvasive marker such as HA are obvious.

The Corgenix HA test kit is an enzyme-linked binding protein assay that incorporates a hyaluronic acid binding protein (HABP) to capture HA. Briefly, diluted patient serum or plasma, controls and reference solutions are incubated in HABP coated microwells (96-well plate) to allow any HA present to react with the immobilized HABP. After washing to remove any unbound materials, HABP conjugated to horseradish peroxidase (HRP) is added to form linkages with the bound HA. Several HABP-HRP conjugates bind to the captured HA molecule, with the number bound proportional to the length of the polymer. After a second incubation and washing, a substrate consisting of tetramethylbenzidine and H_2O_2 (TMB) is added. During this final incubation step, turnover of the TMB by the HRP enzyme causes a color to develop in each microwell proportional to the quantity of HA bound. After incubation, the reaction is terminated by the addition of sulfuric acid followed by a measurement of the color intensity produced in each well. The resulting absorbance value (optical density) of each unknown or control is then compared to a calibration curve constructed from values produced by the calibrators. Unknown results are reported as ng/mL HA (34).

HA Enzyme-Linked Protein Binding Assay

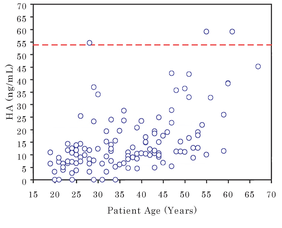


MATERIALS AND METHODS

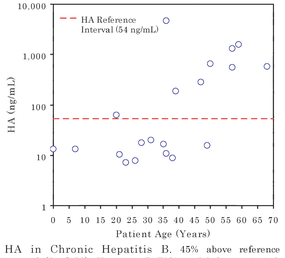
- HA assay kits were provided by Corgenix Inc. (Westminster, CO).
- SPECTRAmax® PLUS plate reader was manufactured by Molecular Devices Corp. (Sunnyvale, CA) and controlled using Molecular Devices Corp. ProMax software.
- Data analysis was performed using Microsoft Excel software.
- Serum and plasma specimens were deidentified using Internal Review Board approved protocols (IRB #7275) and stored short term (< 2 weeks) at 4 - 8 °C or long term frozen at – 70 °C.
- Samples were assayed as instructed by the kit manufacturer.
- Specimen selection criteria for HA concentration in the following groups:
  - Hepatitis C: Virus RNA > log 2.3 IU/mL (detection limit).
  - Acute Hepatitis B: HBsAg and IgM anti-HBc positive.
  - Chronic Hepatitis B: Hepatitis B DNA > 7.8 log copies/mL (reference interval 2.3 log copies/mL).
  - Acute Hepatitis A: Hepatitis A IgM positive.
  - Alpha 1-Antitrypsin Z Phenotype: At least one Z allele.
  - End stage liver disease: Serum albumin < 3.5 mg/dL, total bilirubin > 2.5 mg/dL, prothrombin time > 18.5 sec.
  - Rheumatoid arthritis: Cyclic citrullinated peptide IgG > 20 EU.
  - Normal serum protein, 80 - 90 years: serum protein electrophoresis = normal pattern and concentration, 6.00 - 8.30 g/dL.

RESULTS

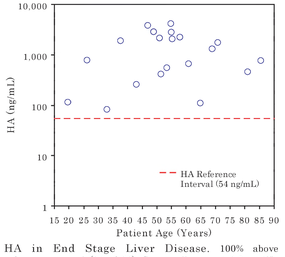
- Limit of detection: 8 ng/mL.
- Analytical measurement range (AMR): 8 - 800 ng/mL.
- Linearity throughout AMR: Slope = 1.008, intercept = 13.64, R^2 = 0.998 (n = 7).
- Within-run precision, three levels (n = 8): 29 ± 0.8, 138 ± 3.4, 546 ± 6.7 ng/mL; CVs of 2.7, 2.5, 1.2% respectively.
- Between-run precision, three levels (n = 7): 44 ± 3.9, 87 ± 7.6, 459 ± 29.3 ng/mL; CVs of 8.8, 8.5, 6.4% respectively.
- Agreement with samples assayed at Corgenix: Slope = 0.957, intercept = –0.56, R^2 = 0.993 by Deming Regression (n = 22, range 29 - 866 ng/mL).
- Kit lot-to-lot variability: Slope = 1.071, intercept = 3.92, R^2 = 0.98 by Deming Regression (n = 22).
- HA stability: 24 hours at room temperature, minimum of two weeks at 4 - 8 °C.



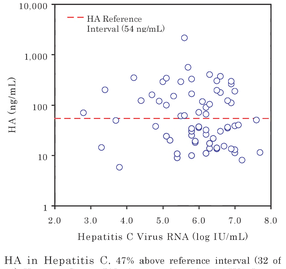
HA Reference Interval Study. Donations from 122 healthy individuals were measured for serum/plasma HA. A reference interval of 54 ng/mL (dashed red line) was established by nonparametric analysis (97.5%)



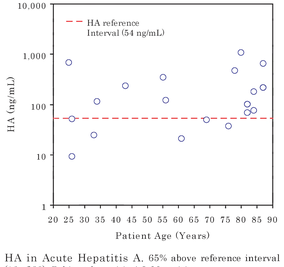
HA in Chronic Hepatitis B. 45% above reference interval (9 of 20). Hepatitis B DNA > 7.8 log copies/mL (Reference interval: < 2.3).



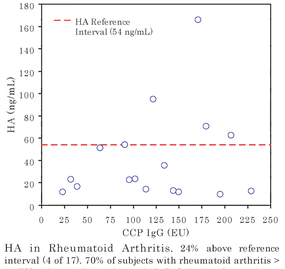
HA in End Stage Liver Disease. 100% above reference interval (20 of 20). Serum albumin < 3.5 mg/dL, total bilirubin > 2.5 mg/dL, prothrombin time > 18.5 sec.



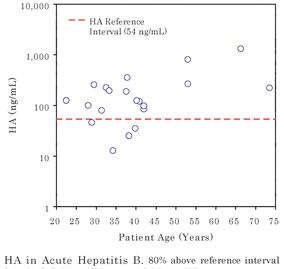
HA in Hepatitis C. 47% above reference interval (32 of 68). Hepatitis C virus RNA detection limit: log 2.3 IU/mL.



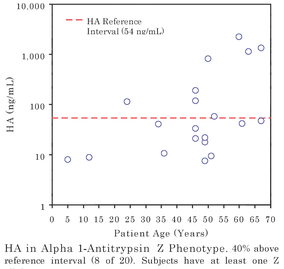
HA in Acute Hepatitis A. 65% above reference interval (13 of 20). Subjects hepatitis A IgM positive.



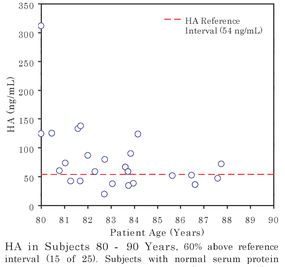
HA in Rheumatoid Arthritis. 24% above reference interval (4 of 17). 70% of subjects with rheumatoid arthritis > 20 EU cyclic citrullinated peptide IgG. Only 2% of normals.



HA in Acute Hepatitis B. 80% above reference interval (16 of 20). Subjects HBsAg and IgM anti-HBc positive.



HA in Alpha 1-Antitrypsin Z Phenotype. 40% above reference interval (8 of 20). Subjects have at least one Z allele.



HA in Subjects 80 - 90 Years. 60% above reference interval (15 of 25). Subjects with normal serum protein electrophoresis patterns and total protein (6.00 - 8.30 g/dL).

CONCLUSIONS

- The Corgenix HA enzyme-linked binding protein assay demonstrates acceptable performance for quantifying HA.
- HA is elevated in a large percentage of individuals with various types of hepatitis and liver disease.
  - Supports possible use in assessing liver fibrosis and cirrhosis in lieu of biopsy.
  - Of greater importance, may reduce biopsies without missing hepatic fibrosis cases.
- HA can be elevated in arthritic and elderly individuals, but not to the degree of those suffering liver complications.

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